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The tumour microenvironment and immune milieu of cholangiocarcinoma

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THE TUMOR MICROENVIRONMENT AND IMMUNE MILIEU OF CHOLANGIOCARCINOMA

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List of abbreviations:

ECM, extracellular matrix

CCA, cholangiocarcinoma

CAF, cancer-associated fibroblast

α-SMA, α-smooth muscle actin

HSC, hepatic stellate cell

EMT, epithelial-to-mesenchymal transition

PDGF, platelet-derived growth factor

PDGFR, platelet-derived growth factor receptor

JNK, c-Jun N-terminal kinase

HGF, hepatocyte growth factor

TGF, transforming growth factor

CTGF, connective tissue growth factor

EGF, epidermal growth factor

SDF-1, stromal cell–derived factor-1

HB, heparin-binding

EGFR, epidermal growth factor receptor

ERK, extracellular signal-regulated kinase

STAT3, signal transducer and activator of transcription 3

CXCR, chemokine (C-X-C motif) receptor

TNF, tumor necrosis factor

TRAIL, tumor necrosis factor-related apoptosis inducing ligand

VEGF, vascular endothelial growth factor

TAM, tumor-associated macrophage

NF-kB, nuclear factor k-light-chain-enhancer of activated B cells

FAP, fibroblast activation protein

MDSC, myeloid-derived stromal cell

BTC, biliary tract cancer

CTLA, cytotoxic T-lymphocyte antigen

iCCA, intrahepatic variant of CCA

PI3K, phosphoinositide 3-kinase

YAP, Yes-associated protein

TAZ, transcriptional coactivator with PDZ-binding motif

SWI/SNF, switching defective/sucrose non-fermenting

MYL9, myosin light chain 9

NLR, neutrophil-to-lymphocyte ratio

TAN, tumor-associated neutrophil

NGAL, neutrophil gelatinase-associated lipocalin

MCP-1, monocyte chemoattractant protein-1

CSF, colony stimulating factor

DC, dendritic cell

COX, cyclooxygenase

iNOS, inducible nitric oxide synthase

TEM, TIE2-expressing monocyte/macrophage

Treg, regulatory T lymphocyte

MHC-I, MHC class I

PD-1, programmed cell death protein-1

TIL, tumor-infiltrating lymphocyte

FoxP3, forkhead box P3

PD-L1, programmed cell death protein-1 ligand-1

LEL-CCA, lymphoepithelioma-like cholangiocarcinoma

EBV, Epstein-Barr virus

EBER, Epstein-Barr virus non coding RNA

CRBP, cellular retinol binding protein

LMP, latency membrane protein

LEL-HCC, lymphoepithelioma-like hepatocellular carcinoma

FGFR, fibroblast growth factor receptor

TCGA, the Cancer Genome Atlas

TKI, tyrosine-kinase inhibitors

Mcl1, myeloid cell leukemia 1

CAR, chimeric antigen receptor

VEGFR, vascular endothelial growth factor receptor

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ABSTRACT

Tumor microenvironment is a complex, multicellular functional compartment that, particularly when assembled as an abundant desmoplastic reaction, may profoundly affect the proliferative and invasive abilities of epithelial cancer cells. Tumor microenvironment comprises not only stromal cells, mainly cancer-associated fibroblasts, but also immune cells of both the innate and adaptive system (tumor-associated macrophages, neutrophils, natural killer cells, and T and B lymphocytes), and endothelial cells. This results in an intricate web of mutual communications regulated by an extensively remodeled extracellular matrix, where the tumor cells are centrally engaged. In this regard, cholangiocarcinoma, in particular the intrahepatic variant, has become the focus of mounting interest in the last years, largely due to the lack of effective therapies despite its rising incidence and high mortality rates worldwide. On the other hand, recent studies in pancreatic cancer, which similarly to cholangiocarcinoma, is highly desmoplastic, have argued against a tumor-promoting function of the tumor microenvironment. In this review, we will discuss recent developments concerning the role of each cellular population and their multifaceted interplay with the malignant biliary epithelial counterpart. We ultimately hope to provide the working knowledge on how their manipulation may lead to a therapeutic gain in cholangiocarcinoma.

HIGHLIGHTS

- Cholangiocarcinomas, including the intrahepatic and perihilar anatomical subtypes, are characterized by a prominent stromal reaction.
- In cholangiocarcinoma, the tumor microenvironment is populated by a heterogeneous plethora of cells, including not only stromal cells (mainly cancer-associated fibroblasts), but also innate immune cells (tumor-associated macrophages, neutrophils), and adaptive immune cells (tumor-infiltrating lymphocytes).
- Deciphering the complex interactions between malignant cholangiocytes and cells hosted in the tumor microenvironment is key to uncover novel therapeutic interventions targeting single cell compartments of the tumor microenvironment, in support of tumor cell–specific targeted therapies.
- Targeting stromal and immune cells may be relevant strategies to halt cholangiocarcinoma progression.

Introduction

The development of a highly reactive microenvironment in conjunction with the growth of the tumor mass is a functional hallmark of many epithelial cancers with pronounced invasiveness and shortage of therapeutic options (1). The tumor microenvironment is a heterogeneous, 'multiethnic' compartment, encompassing stromal cells, in particular activated fibroblasts (so-called cancer-associated fibroblasts), and endothelial cells, along with a crowd of innate and adaptive immune cells (tumor-associated macrophages, neutrophils, natural killer cells, and T and B lymphocytes), which act in concert to provide tumor cells with a plethora of pro-invasive cues. In addition, extracellular matrix (ECM) degradation and remodeling support and encourage the reciprocal interactions among the different cell populations, thereby contributing to a pleomorphic milieu proficient to tumor growth and invasion. The result is the generation of a complex network of intercellular crosstalk. In other words, within this 'ecosystem', the non-malignant stromal and immune cell elements represent the 'soil' where the 'seed', namely the malignant epithelial counterpart, is not only hosted, but also nourished, aiding its engraftment and overgrowth (2).

In cholangiocarcinoma (CCA), including both the intrahepatic and the perihilar anatomical subtypes, the extent of the tumor stroma is so prominent that it outweighs the tumoral component (3). Other epithelial malignancies of glandular origin, including breast, prostate, gastric and pancreatic adenocarcinomas, feature an abundant desmoplasia, but the effects can be different, depending on the specific disease context. Many studies highlighted the pro-tumorigenic role played by the tumor microenvironment, and the classic view supports the concept that targeting tumor stroma may offer a valuable strategy to halt tumor progression (4). In contrast with tumor cells, whose genetic heterogeneity makes response to conventional chemotherapy unpredictable, stromal and immune cells are not transformed, and thus, offer a therapeutic advantage, as they display a much more predictable response to therapy (5). From this point of view, anti-angiogenesis therapy, pioneered as an approach in some settings such as metastatic colorectal cancer, has shown to be effective (6). However, recent data derived from experimental models argue against the pro-invasive functions of the stromal reaction. In mouse models of pancreatic ductal adenocarcinoma, indeed, depletion of cancer-associated fibroblasts induced a more aggressive tumor phenotype and accelerated tumor spread with reduced survival, thus indicating that some stromal elements may act to restrain rather than stimulate tumor growth (7, 8). Alternatively, homeostatic restoration of the desmoplastic stroma by reprogramming fibroblasts into their quiescent state is an effective strategy to slow progression of pancreatic cancer (9, 10). The aim of this review is to clarify the role of the tumor microenvironment in CCA, and to understand if it can provide targets for therapeutic intervention. Therefore, we will examine the role of each cell compartment and its intricate interplay with the tumoral cells, at instances by highlighting results from other desmoplastic epithelial cancers, before discussing if their manipulation may lead indeed to a therapeutic gain.

The role of cancer-associated fibroblasts in CCA

A major cellular population of the desmoplastic stroma of CCA is represented by fibroblast-like cells, called cancer-associated fibroblasts (CAFs). These cells are activated myofibroblasts, expressing α-smooth muscle actin (α-SMA) (Figure 1A) (11, 12). Most observations indicate that CAFs play a key role in mediating CCA growth and progression, as well as resistance to therapy. Hence, high α-SMA expression in the tumor stroma correlates with poor survival in patients with CCA (13, 14). Important evidence of their role was provided by the demonstration that triggering CAF apoptosis with the BH3 mimetic navitoclax reduces tumor burden and metastasis *in vivo* in a syngeneic rat model of cholangiocarcinoma (15).

Mechanisms underlying CAF recruitment

CAFs constitute a phenotypically heterogeneous group of myofibroblasts, whose origin is still uncertain and probably multiple (2). For instance, distinct subpopulations of CAFs expressing specific cell surface markers such as podoplanin, a mucin-like transmembrane glycoprotein (16) or CD10, a cell surface metalloprotease (17), have been associated with lymphatic spread or with different anatomical location, respectively. Immunohistochemistry studies using cell-type specific markers have reported that these cells most likely originate from hepatic stellate cells (HSCs) (14) and/or portal fibroblasts (18, 19). Other potential cellular sources of CAFs include bone marrow-derived mesenchymal cells, which are recruited from the peripheral blood (20). On the other hand, the contribution of epithelial-to-mesenchymal transition (EMT) of cholangiocytes to myofibroblasts has been refuted in murine models of liver fibrosis using lineage-tracing techniques (21-23). Furthermore, *in vivo* xenotransplant studies with CCA cells demonstrated that CAFs are not generated through an EMT process of CCA cells, but rather their recruitment was regulated via platelet-derived growth factor (PDGF)-D secretion by CCA cells. PDGF-D promotes fibroblast migration through its cognate receptor PDGFRβ, and activation of its downstream effectors, Rho GTPase and c-Jun N-terminal kinase (JNK) (24). In a murine model of breast cancer, also a highly desmoplastic tumor type, DNA damage in tumor cells during tumor initiation induces activation of fibroblasts via COX-2/prostaglandin E2 and activin-A (25). This pathway has not been explored in CCA.

Cross-talk between CAF and tumor cells In the past years, the characterization of biliary epithelial cells and stromal cells from human surgical CCA resected specimens has provided new information on their crosstalk with CCA tumor cells and other immune cell types in the tumor stroma (24, 26). CAFs are pivotal in this context as they are able to communicate in a multi-directional manner with virtually every cell type in the tumor microenvironment (27). The molecular regulation of this communication is rather challenging to dissect due to its high level of complexity, plasticity and dynamics (28). CAFs are able to enhance the malignant phenotype of CCA cells via various soluble factors, e.g. hepatocyte growth factor (HGF), transforming growth factor (TGF)-β, connective tissue growth factor (CTGF), epidermal growth factor (EGF), stromal cell–derived factor-1 (SDF-1) and

angiotensin II, secreted in conjunction with major ECM components and matrix metalloproteases (MMPs) (11). In turn, CCA cells are capable of attracting and activating fibroblasts or myofibroblast precursor cells, e.g. via PDGF-D and TGF-β (24, 29). The presence of a reciprocal paracrine loop between CAFs and tumor epithelial cells mediated by the heparin-binding (HB) EGF/EGF receptor (EGFR) axis is paradigmatic of the intense two-ways communication by which CAFs sustain invasiveness of CCA cells and in turn, are persistently activated by them. CAFs produce HB-EGF, which activates EGFR, expressed by CCA cells. Following activation, EGFR signals via its downstream effectors, extracellular signal-regulated kinase (ERK) 1/2 and signal transducer and activator of transcription 3 (STAT3), leading to nuclear translocation of β-catenin, which unfolds a transcriptional program involved in cell motility and invasion (29). Activation of EGFR signaling also triggers TGF-β1 production by CCA cells, which further enhances myofibroblast activation and CAFs synthesis of HB-EGF (29). Similar to HB-EGF, CAF-derived SDF-1 stimulates CCA cell invasion acting via ERK1/2 and AKT upon binding to its receptor chemokine (C-X-C motif) receptor 4 (CXCR4), and this effect is abrogated by the CXCR4 inhibitor AMD3100 (30). PDGF-B is another important paracrine signal emitted by CAFs and influencing CCA cell behavior. Once secreted by CAFs, PDGF-B interacts with its cognate receptor PDGFR-β expressed by CCA cells to induce tumor cell resistance to tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) by activating the Hedgehog signaling. This paracrine mechanism has translational relevance, as shown in an orthotopic syngeneic rat model of CCA, where Hedgehog inhibition by cyclopamine reduces tumor growth by stimulating CCA cell apoptosis (31). Recent data demonstrate that in addition to promoting CAFs accumulation within the tumor stroma, PDGF-D produced by CCA cells provides CAFs with pronounced pro-lymphangiogenic functions, mediated by secretion of vascular endothelial growth factor (VEGF)-A and VEGF-C, which induce the chemotaxis of lymphatic endothelial cells to gather in a proper vascular bed also favoring CCA cell intravasation (Figure 1C-D). Furthermore, in a syngeneic rat model of CCA, depletion of CAFs by navitoclax reduces the lymphatic vascularization of the tumor mass and more importantly, lymph node metastases *in vivo* (32). Accordingly, transcriptomic analysis of the tumor stroma in CCA found that a stromal signature enriched for TGF-β and TNF receptor superfamiles, associated with a strong expression of pro-inflammatory mediators, significantly correlated with poor prognosis (33). Taken together, these findings point towards an important tumor-supporting role of CAFs already at very early time points during tumor evolution.

Cross-talk between CAF and innate immune cells Beside cancer cells, CAFs communicate extensively with cells of the immune system, including tumor-associated macrophages (TAMs). Monocytes and macrophages are of critical importance for the activation of fibroblasts, as originally noted by Ross studying skin wound healing (34). In murine models of liver fibrosis, hepatic macrophages promote disease progression via nuclear factor k-light-chain-enhancer of activated B

cells (NF-kB)-dependent enhancement of HSC survival (35). In turn, CAFs have been shown to recruit macrophages in various murine and human tumors, thereby stimulating angiogenesis and tumor progression (36). While these results and the significant (molecular and cell biological) overlap between wound healing, fibrosis and tumor formation (37) argue for a significant role of the CAF-macrophage axis in CCA, functional studies addressing this intriguing topic are missing. Besides cells of innate immunity, a large body of evidence supports a pivotal role of CAFs in the regulation of adaptive immunity in the tumor microenvironment. The vast array of cytokines, chemokines and pro-angiogenic factors secreted by CAFs, is believed to predominantly generate an immunosuppressive microenvironment (12). Compared with myofibroblasts, CAFs also express high levels of fibroblast activation protein (FAP), a membrane-bound serine protease implicated in ECM remodeling, and FAP overexpression has been reported in tumor stroma of highly invasive epithelial cancers, as pancreatic ductal adenocarcinoma (38). Interestingly, FAP expression identifies a subset of CAFs with upregulated expression of proinflammatory genes, which promotes immunosuppression by recruiting myeloid-derived stromal cells (MDSCs) in the tumor microenvironment via STAT3–CCL2 signaling (39). The immune-suppressive function of CAFs was convincingly demonstrated by immunogenic tumor (and stromal cell) necrosis in response to genetic ablation of a CAF-subset in murine pancreatic adenocarcinoma (40). Of note, two independent groups reported a rather unexpected tumor progression upon CAFs depletion in murine tumor models, demonstrating that the consequences of CAFs depletion are highly context-dependent (7, 8). With respect to CCA, a small study analyzing immune-related transcripts in resected biliary tract cancers (BTCs) displayed a significant association between the expression of cytotoxic T-lymphocyte antigen (CTLA)-4 and relapse-free survival (41). While these results are interesting and suggest an immunosuppressive environment promoting tumor progression, the functional relevance and molecular mechanisms of the cross-talk between CAFs and adaptive immune cells remains largely elusive in CCA. This topic will conceivably become of growing interest in the era of immunotherapy of solid tumors.

Effects of CAF on ECM

Alongside the multitude of soluble factors enabling communications with the different cell types that populate the tumor microenvironment, CAFs produce major ECM components, such as tenascin C and periostin, and secrete several MMPs. Coupled with those released by the cancer cells themselves, MMPs are essential to degrade and remodel the ECM, as pre-requisite for tumor progression. In CCA, CAFs express MMP1, MMP2, MMP3, and MMP9 and this phenotype is associated with tumors that are more aggressive (42, 43). However, recent data indicate that in desmoplastic tumor microenvironments, CAFs can make passageways in the ECM in an MMP-independent manner. The basement membrane is the barrier that at the stage of carcinoma *in situ*, segregates tumor cells from the stroma, and it must be broken to let tumor cell spread through the

surrounding tissues. In addition to proteolysis, rupturing of the basement membrane can be favored by fine CAF movements dependent upon their overdeveloped contractility. By pulling and stretching the basement membrane, CAFs exert mechanical forces which soften the barrier integrity and lead to the formation of gaps permissive for cancer cell migration and invasion, as elegantly shown in an *ex vivo* model of colorectal cancer (44).

The evolving role of the ECM in CCA

As discussed above, in intrahepatic and peri-hilar CCA, neoplastic bile ducts are tightly surrounded by an abnormally remodeled and stiff ECM, which contributes to tumor invasiveness and progression. Similar to the epithelial part, the ECM gradually undergoes a phenotypic switch from a thin layer beneath the basal side of the normal biliary epithelium into a thick and rigid structure favoring tumor duct interactions with many stromal and immune cells (2). The native structure of ECM is perturbed by deposition of new structural components and its concurrent dismantlement by proteases, either secreted by cell types recruited in the tumor microenvironment, in particular CAFs, TAMs, as well as by the tumoral cells themselves (23). Some major ECM constituents, such as tenascin-C, osteopontin, and periostin, are newly synthetized in CCA, where they promote key tumor properties, as invasive cell growth, chemoresistance, and metastatic spread (45, 46). Moreover, their overexpression, mainly at the invasive front as in the case of tenascin-C, correlates with an increase in tumor size, lymph node metastatization, and a worsen outcome (46). Periostin, in particular, has drawn increasing interest as a potential prognostic biomarker and putative molecular target in the intrahepatic variant of CCA (iCCA) (45). Periostin is a glycoprotein belonging to the TGF-β family-inducible matricellular proteins, extensively represented also in the ECM of other desmoplastic epithelial cancers, as reported in pancreatic ductal adenocarcinoma (47). In desmoplastic tumors, CAFs are the main cell source of periostin. In malignant cholangiocytes, periostin interacts with other ECM components, particularly collagen type I and tenascin-C, and with integrins, particularly α5β1, α5β3, α5β5, and α6β4**,** leading to activation of a proliferative cascade mediated by phosphoinositide 3-kinase (PI3K)/AKT signaling (48). Of note, CCA cells express the periostin receptor, the α5 subunit of integrin, and knockdown of α5 integrin decreased tumor proliferation and invasion (49). Gene expression profiling of laser-capture microdissected stroma obtained from human iCCA revealed two additional ECM components, laminin and osteopontin, that besides being markedly up-regulated compared with non-tumor tissue, had also strong clinical relevance as they significantly correlated with poor prognosis (50). In particular, stromal overexpression of osteopontin and TGF-β2 were the most significant independent predictors in terms of both overall and disease free survival (50). The ‘desmoplastic’ ECM can play a pro-tumorigenic effect also thanks to its increased rigidity. External mechanical forces induce cells to change the tension and the structure of the cytoskeleton, exerting potent tumor suppressor functions in normal epithelia. Two intracellular mechanosensors, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), are

particularly sensitive to ECM stiffening and act as fundamental supervisors of both tissue repair mechanisms and tumor initiation and progression by regulating crucial cell functions, including proliferation, survival, plasticity, and invasion (51). Once activated in tumor epithelial cells by nuclear translocation and interaction with the transcription factor TEAD, YAP/TAZ elicit a number of pro-invasive pathways, such as the mTOR/cyclin D1-mediated hyper-proliferation, the AKT-mediated escape from apoptosis, and the activation of the EMT program endowing tumoral cells with mesenchymal properties (52). Recent data show that alternative to association with TEAD, YAP/TAZ nuclear activity is inhibited by its association with the switching defective/sucrose non-fermenting (SWI/SNF) chromatin-remodeling complex through ARID1A (53), whose genetic inactivation has been reported in about 7% of iCCA (54). The association between ARID1A–SWI/SNF and YAP/TAZ is finely regulated by cellular mechanotransduction: whereas soft ECM favors YAP/TAZ inhibitory sequestration within the ARID1A-containing SWI/SNF, conversely stiff ECM induces YAP/TAZ detachment from SWI/SNF and their binding to TEAD (53). Notably, a stiff ECM enhances the activity of YAP/TAZ not only in cancer cells but also in stromal cells, including CAFs. Active nuclear YAP was expressed by CAFs, and YAP depletion in CAFs reduced their tumor-promoting functions. In breast cancer, ECM stiffening regulates a feed-forward self-reinforcing loop that helps to maintain the CAFs phenotype by sustaining YAP activation. In turn, YAP controls the expression of cytoskeletal regulators, including ANLN and DIAPH3, and of the myosin light chain 9 (MYL9) that regulates CAFs contractility and motility (55).

The role of the innate immune system in CCA

Several innate immune cells encompassing macrophages, neutrophils, and natural killer (NK) cells are present in the tumor microenvironment and they significantly affect cholangiocarcinogenesis (Figure 1B). Tumor-associated macrophages (TAMs) are the most relevant infiltrating immune cell population within the tumor microenvironment (56). High tissue macrophage density has been associated with poor prognosis of patients with CCA (57). Furthermore, circulating $CD14^+/CD16^+$ monocyte levels correlate with TAM infiltration and are associated with poor prognosis (58). CCA cells induce macrophage polarization toward the alternatively activated macrophage or M2 phenotype via the STAT3 pathway, these being associated with bad prognosis in patients with CCA (59). Macrophages, through their crosstalk with CCA cells participate in tumor growth by releasing a variety of inflammatory, growth, and proliferative factors (60-62).

Elevated preoperative peripheral blood neutrophil-to-lymphocyte (NLR) ratio is also a poor prognostic factor for intrahepatic and extrahepatic CCA (63-66), as well as in patients with advanced CCA undergoing chemotherapy (67, 68). Distribution of tumor-associated neutrophils (TAN) in CCA tissue sections by immunohistochemical analysis of CD15, a marker of mature granulocytes, has revealed that patients with high CD15 expression have shorter disease free survival time and overall survival than those with low expression (69). Moreover, neutrophil gelatinase-associated lipocalin

(NGAL) expression in bile has been identified as a valuable candidate to discern malignant from benign biliary strictures (70).

NK cells are innate lymphocytes with the capacity to recognize and eliminate tumor cells via the release of cytotoxic granules (71). This recognition is regulated by a plethora of activating and inhibitory immune receptors expressed on the surface of NK cells. With these, NK cells can sense and respond to 'stressed' cells, such as cancer cells, in the nearby area (71). The liver is enriched in NK cells compared with other lymphocytes and they represent up to 30-40% of all liver lymphocytes (72). Despite this, little is known regarding NK cells in CCA. Instead, more studies have been performed in HCC where several immune receptors expressed by NK cells, such as CD96 and NKp30, have been associated with better prognosis (73, 74). Furthermore, immunotherapy with infusion of activated allogeneic NK cells have also been performed in HCC patients with promising outcomes (75, 76). However, before such treatments can be employed in CCA, it is necessary to determine if NK cells have the capacity to infiltrate CCA, which NK cell receptor–ligand interactions are important for recognition of CCA, and how NK cells are affected by evasion strategies employed by the tumor and its microenvironment.

Mechanisms underlying innate immune cell recruitment The mechanisms underpinning the complex innate immune response in CCA are still largely unknown. As mentioned above, macrophage polarization towards the tumor-promoting M2 state is associated with poor prognosis and metastasis in CCA (77). TAMs are a subtype of M2 macrophages with particular powerful tumor-promoting functions (78) and derive mainly from $CD14^+/CD16^+$ circulating monocytes rather than from resident macrophages; indeed, the massive expansion of intrahepatic macrophages observed during chronic liver injury follows the influx of circulating monocytes (79). Monocyte recruitment into the liver is promoted by chemoattractant molecules, including monocyte chemoattractant protein-1 (MCP-1/CCL2), colony stimulating factor (CSF)-1, and VEGF-A (80). Notably intrahepatic macrophages are an important source of CCL2 that stimulates the migration of bone marrow-derived monocytes (81). Further, macrophage recruitment is supported by epithelial tumor cells and CAFs, and is also stimulated by regulatory pathways (Notch, IL6/STAT3, PI3K) and specific cytokines (IL1β, IL10, IL13, and IL4) (82). In CCA, a stem cell-like compartment is particularly active in promoting recruitment of circulating monocytes along with their differentiation into TAMs, by releasing IL13, IL34, and osteoactivin. Importantly, TAMs associated with the cancer stem cell niche display unique features, including expression of both M1 and M2 phenotypic traits, increased adhesive and invasive capabilities, *in vitro*, and enhanced tumor-promoting activities, *in vivo* (83). This has lent support to the notion that different TAM subsets are present within the tumor, reflecting different hints derived from various cell niches. Finally, TAMs themselves modulate the CCA microenvironment by secreting TNF-α, TGF-β, IL6, IL10, and VEGF-A (61), which support EMT, tumor growth, and metastasis.

Infiltration of TANs has also been associated with poor prognosis in CCA (69). Recruitment of neutrophils in CCA is predominantly driven by CXCL5, which has direct chemoattractant effects on TANs *in vitro* through PI3K-AKT and ERK1/2 signaling pathways (84). TANs expressing CCL2 and CCL17 recruit TAMs and regulatory T lymphocytes, eventually generating an immunosuppressive environment, which sustains tumor promotion.

As aforementioned, CAFs are highly active in recruiting innate immune cells, and can play a dual role with both tumor-suppressive and tumor-promoting potential that may be partly explained by the regulatory state(s) and heterogeneity of CAFs. Beside recruiting immunosuppressive MDSCs and TAMs (39), CAFs attract and educate dendritic cells (DCs) into a regulatory state, attenuating the expression of antigen-presenting HLA molecules, reducing the capability to attract and activate tumor infiltrating lymphocytes (85), and enhancing the ability of MDSCs to inhibit T cell proliferation via FAP/STAT3/CCL2 axis (39).

Mechanisms whereby innate immune system influences tumor growth

Well in line with the above outlined heterogeneity and complexity of innate immune cells in tumor microenvironment, the mechanisms by which these cells impinge on tumor progression are diverse. TAMs are able to accelerate tumor progression on multiple levels. They take part in tumoral angiogenesis via the secretion of pro-angiogenic (e.g. VEGF-A, angiopoietin, IL8) and pro-inflammatory mediators, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), supporting tumor growth beyond the limits of oxygen and nutrient diffusion (86). Additionally, macrophage-derived Wnt ligands, such as Wnt3a and Wnt7b, activate canonical Wnt pathway, contributing to CCA cell proliferation (61, 62). Thus, macrophage depletion or Wnt signaling inhibition halts tumor growth *in vitro* and in experimental models recapitulating CCA (61, 62). Furthermore, TAMs are able to dampen the efficacy of anti-proliferative drugs against solid tumors in a substantial manner. Certain chemotherapeutic agents, e.g. doxorubicin, enhance TAM accumulation in the tumor microenvironment, ultimately attenuating their cytotoxic effects (87). Although the molecular underpinnings are still enigmatic (88), recent work by Lyssiotis' group showed that in murine models of pancreatic ductal adenocarcinoma, pyrimidine species released by TAMs inhibit gemcitabine via functional interference with drug uptake and metabolism (89). This suggests that TAM metabolism is interwoven with that of cancer cells with the potential to modulate the therapeutic response of solid tumors. Anti-tumor immunity is another paramount effect played by TAMs. TAMs can suppress tumor-inhibiting T cells via different mechanisms, e.g. via depletion of essential metabolic precursors such as L-arginine (90) by hypoxia-mediated upregulation of arginase and iNOS (91).

Within the macrophage population, the TIE2-expressing monocytes/macrophages (TEMs) represent a myeloid cell subset found in blood as well as in tumor tissue with relevance for tumor progression. TIE-2 is the receptor for angiopoietins, and TEMs are indeed highly pro-angiogenic (92). Furthermore, TEMs, via IL10, suppress T cell proliferation, increase the CD4/CD8 ratio, and support

the expansion of $CD4^+CD25^{high}FOXP3^+$ regulatory T lymphocytes (Tregs), highlighting TEMs as a vigorous immunosuppressive force in the tumor microenvironment (93). However, in CCA TEM abundance is associated with improved prognosis, suggesting additional, hitherto unknown mechanisms by which TEMs inhibit tumor progression (94).

In the innate immune system, NK cells are of pivotal importance given their ability to control microbial infections and tumor progression (71, 95, 96). Phenotypically, NK cells are defined as $CD3^-CD56^+$ lymphocytes in humans and as $CD3^-NK1.1^+$ lymphocytes in mice. Lysis of target cells, including neoplastic cells, is the hallmark function of NK cells and of paramount importance for their tumor-inhibiting efficacy (71). Anti-tumor effects of NK cells can be overcome by various means, e.g. conservation of MHC class I (MHC-I) expression, shedding of ligands for the activating NK receptor (e.g. NKG2DLs), and secretion of immunomodulatory molecules (e.g. TGF-β, prostaglandin E2, adenosine) by tumor cells, ultimately resulting in tumor progression (71). Interestingly, under defined conditions, NK cells can express programmed cell death protein-1 (PD-1) and CTLA-4, which are instrumental for anti-tumor T cell responses and represent targets for several approved immunotherapy agents (97). The functional relevance of NK cells for CCA is only beginning to emerge. *In vitro*, activated NK cells have been shown to enhance the cytotoxic efficacy of cetuximab against human CCA cell lines (98), while *in vivo*, infusion of *ex vivo*-expanded human NK cells in HuCCT-1 xenografts in nude mice displayed significant tumor-inhibiting effects (99).

The role of adaptive immune system in CCA

Convincing evidence in both mouse models and human patients support the ability of the adaptive immune system to identify and target arising tumor cells, and thus, to behave as a primary defense against cancer (100). Tumor-infiltrating lymphocytes (TILs) are present in many solid tumors and form highly heterogeneous populations (101). While TILs can act against tumor cells to inhibit carcinogenesis and to hamper cancer progression ('immune surveillance'), cancer cells devise stratagems to circumvent anti-cancer immune reactions and boost tumor progression ('immune escape') (102, 103). Tumor infiltrates include B lymphocytes, $CD8^+$ cytotoxic T lymphocytes, cytokine secreting $CD4^+$ T helper lymphocytes, and Forkhead box P3 $(FoxP3)^+$ Tregs. Additionally, DCs as an important bridge between adaptive and innate immune responses are abundant in the tumor microenvironment and shuffle antigen towards the draining lymph node for immune activation (104). In CCA, $CD8^+$ T lymphocytes have been studied in terms of presence and location within the tumor. Overall, $CD4^+$ TILs prevail in the peritumoral region (105), while $CD8^+$ TILs are mostly prevalent in the intratumoral tissue (105, 106). More than half of resected CCAs are positive for $CD8^+$ TILs, of which 30% are reported positive for Granzyme B, indicating an activated and cytotoxic phenotype (107). Multiple studies confirm that enhanced $CD4^+$ and $CD8^+$ infiltrates (also in combination with low numbers of macrophages) in CCA and extrahepatic BTCs are associated with better overall survival, fewer lymph node metastases and reduced venous and perineural invasion (106-110),

whereas low numbers of $CD8^+$ TILs are associated with poor overall survival (111). In addition, MHC-I expression in intrahepatic and extrahepatic CCA strongly correlates with the $CD4^+$ and $CD8^+$ tumor infiltrate and is associated with longer overall survival (112). Consistent with these findings, in BTCs, the total count of lymphocytes of the adaptive immune response showed a stepwise decrease in invasive and metastatic tumors compared with non-invasive precursors (106), suggesting a gradually developing immune escape of the tumor.

The number of $CD4^+$ and $CD8^+$ lymphocytes in CCA tissue may additionally be influenced by DCs. It has been demonstrated that immature $CD1a^+$ DCs reside only in the tumor core, while mature $CD83^+$ DCs are found predominantly at the invasive front (110). Moreover, the number of DCs at the invasive margin correlated with the number of $CD4^+$ and $CD8^+$ TILs in the tumor bulk. Additionally, mature DCs surrounded by $CD4^+$ and $CD8^+$ cells are observed at the cancer periphery, highlighting the importance of immune cell interactions in CCA. A similar finding was reported in colorectal carcinoma, indicating that these clusters of DCs and T lymphocytes are formed to maximize T cell activation against the tumor (110, 113). Takagi suggested a direct link between the abundance of mature DCs able to prime anti-tumor T cells at the invasive margin and the risk of cancer invasion and metastasis (110). Indeed, they could show that $CD4^+$ and $CD8^+$ T cell infiltration in the cancerous tissue is enhanced by mature $CD83^+$ DCs at the tumor-host interface of CCA, with $CD83^+$ patients displaying a better prognosis and lower incidence of lymph node metastases than $CD83^-$ patients (110). Furthermore, patients classified with an advanced tumor stage showed significantly lower numbers of either immature or mature DCs.

While several studies have drawn attention to T cells, the role of B lymphocytes in CCA is still far from clear. B cells have been identified in TIL populations in BTC, but they are only rarely observed in patient tissues (105, 106). Albeit high densities of $CD20^+$ cells have been observed in low-grade tumors and associate with a favorable overall survival (106), future studied are needed to clarify their relevance.

Mechanisms of immune surveillance and immune escape

The presence of immunogenic tumor-associated antigens has been demonstrated in CCA patients (114). Importantly, the cytotoxic reaction is balanced by immunosuppressive signals. Indeed, CCL2 secreted by tumor cells, TAMs, and CAFs, stimulates tumor-infiltrating T cells to acquire CD4/CD25 expression and become Tregs (115). Tumor-associated Tregs secrete IL10 and TGF-β, which inhibit cytotoxic T cells and NK cells and shape an immunosuppressive milieu. Further, Tregs bind IL2, making it unavailable in the tumor microenvironment and thus preventing the activation of additional immune cells (116). A recent study confirmed that CCA cells also activate natural Treg-like $CD4^+CD25^-$ cells, leading to an increased expression of TGF-β, which suppresses the immune response (117). TGF-β is overexpressed in CCA, and correlates with poor prognosis, lymph node and distant metastases, and tumor recurrence (118). However, TGF-β signaling also conveys a

tumor-suppressing influence by inhibiting tumor growth in the early stage of malignant transformation (119).

FoxP3 is a distinctive feature of Tregs but it is overexpressed also by tumor cells (120). Knockdown of FoxP3 in tumor cells *in vitro* reduced proliferation and invasiveness in CCA cells, inhibited T cell survival, and reduced IL10 and TGF-β signaling in the tumor microenvironment (120). Consequently, FoxP3 overexpression correlates with lymphatic metastasis, poor survival and shorter disease-free survival (111, 120). Furthermore, FoxP3 overexpression is accompanied by CTLA-4 overexpression (41). Indeed, CTLA-4 is expressed on the surface of Tregs and has to bind to CD80 on antigen-presenting cells to exert inhibitory effects on cytotoxic cells (112). Interestingly, a deregulation of genes related to immune modulation in BTCs was more pronounced in the peritumoral than in the tumor tissue and facilitated tumor recurrence and chemo-resistance. Strong CD80 expression, likely reflecting the enrichment of activated Tregs in the microenvironment, correlated with resistance to adjuvant chemotherapy. Furthermore, the expression of CTLA4 in the peritumoural area has prognostic value highlighting the concept that immune escape in CCA associates with poor prognosis (41).

In order to evade immune surveillance as mechanism of resistance, cancer cells frequently manipulate immune checkpoints such as PD-1 and CTLA-4, that once activated by their specific ligands (PD-L1 and CD152, respectively), promote peripheral T cell exhaustion. High expression of PD-L1 among other immune checkpoints and of tumor-specific neoantigens characterized a subset of CCA patients (5.9%, 14/239) with high mutational load and poor prognosis (121). Both PD-1 and PD-L1 are up-regulated in neoplastic cells (122-124), and overexpression is associated with increased invasiveness, poor outcome, and worse disease- and metastasis-free survival, especially when accompanied by low $CD3^+$ or $CD8^+$ infiltrate (109, 122, 125, 126). Conversely, low PD-L1 expression (in combination with high MHC-I expression) was found to be related to favorable prognosis (127). Consequently, the PD-L1/PD-1 pathway might be responsible, to some extent, for lymphocyte apoptosis in CCA progression and account for an increased cancer's malignant potential.

Notch signaling, an important morphogen in the liver, and a signaling mechanism associated with iCCA (128, 129), can also modulate the immune cell regulation necessary for activation of T-helper 1 cells (130) and $CD4^+FoxP3^+$ Tregs (131). In addition, Notch may contribute to M1 polarization of macrophages and to their relationships with CAFs. Since Notch is also involved in T cell induction and in stimulating T cell effector secretory functions (IL10, IL22, and IFN-γ), it is tempting to hypothesize that Notch is crucial for directing T cell infiltrates in CCA (130, 131).

The interaction between the immune system and the neoplastic epithelial cells: lesson from the lymphoepithelioma-like CCA

The lymphoepithelioma-like CCA (LEL-CCA) is a variant of iCCA with distinct epidemiological, morphological, and clinical features. So far, 40 cases have been described, the majority in women

from South-East Asia. In spite of this rarity, LEL-CCA is of interest because it represents a peculiar model of interaction between the immune and neoplastic compartment, and is characterized by significantly superior overall survival when compared with classical iCCA of corresponding stage (132-134). Histologically, LEL-CCA consists of undifferentiated epithelial cells and dense polyclonal lymphocyte infiltrate but in absence of a typical stromal reaction. Tumour cells are arranged in sheets and express the pankeratin A1/A3 and the biliary type cytokeratin K7 and K19 (135); markers of stemness such as CD133 and EpCAM are frequently expressed. The lymphoid infiltrate includes $CD3^+$ and, to a lesser extent, $CD20^+$ cells, and interestingly, metastatic lesions of LEL-CCA lose the lymphoid component. Epstein-Barr virus (EBV) non coding RNA (EBER) is present in almost all cases (136). Although genetic changes of LEL-CCA are unknown, the ability of EBV to induce epigenetic changes resulting in cell proliferation and oncogenesis is well recognized. Accordingly, LEL-CCA is characterized by DNA hypermethylation, in particular of cellular retinol binding protein-I (CRBPI) and of cellular retinol binding protein-IV (CRBPIV), significantly more frequent than in classical iCCA (132). The type of EBV latency in LEL-CCA has been elucidated only in part. EBERs were positive in almost all cases, latency membrane proteins-1 and -2 (LMP1 and LMP2) were negative in eight tested samples, and LMP-related gene showed a 30 bp deletion in two tested cases (137, 138). Thus, similar to nasopharyngeal carcinoma, the expression of EBV-related antigens and tumour genetics might drive the lymphocyte recruitment. Expression of PD-L1 has been studied in LEL-CCA and compared with iCCA, showing a much higher rate in LEL-CCA in both tumor and tumor-infiltrating cells, though the latter were not specifically phenotyped (124, 139). In theory, these findings challenge the hypothesis that PD-L1 is associated with a poor prognosis. However, in lymphoepithelioma-like hepatocellular carcinoma (LEL-HCC), where strong PD-L1 expression was similarly reported, the infiltrating cells mostly consist of T cells, and the ratio of $CD8^+$ to $FoxP3^+$ Treg cells is high (140), suggesting that in this setting, a favourable long-term outcome is not at odds with an up-regulation of PD-L1. It remains to be evaluated whether the same holds true in LEL-CCA.

Modulating each single cell compartment for therapeutic gain in iCCA

In the last years, technological advances such as next-generation sequencing (NGS) have unraveled the high genomic and transcriptomic heterogeneity of iCCA, uncovering promising molecular targets for therapeutic intervention (121, 141, 142). While therapy targeting the cancer cells becomes increasingly more individualized, the contribution of the tumor microenvironment especially in highly desmoplastic tumors appears now clearer. The number of molecular biomarkers derived from each cell compartment of the tumor microenvironment holding prognostic value in CCA are summarized in Table 1. Furthermore, given the uniform reactive phenotype, the tumor stroma is additionally, an attractive therapeutic target. In this regard, promising strategies include molecular targeting of tumor cells, CAFs, immune cells, and vascular cells (Table 2). Among tumor cell targeted therapy, we will discuss only those related to *fibroblast growth factor receptor (FGFR)* mutations as

recently turned-out to be potentially relevant also for the modulation of the microenvironment. A comprehensive review of the novel mutation-based tumor cell targeted strategies are outlined in the specific chapter of the present special issue, which the reader may eventually refer to.

Tumor cell targeted therapy Within the last decade, the knowledge of molecular subtypes of CCA expanded remarkably. Identification of druggable targets and candidate molecules is gaining traction based on NGS. Exploiting *FGFR* mutations in CCA is one of the most advanced, promising approaches, together with therapies directed against EGFR, especially Her-2 mutations and IDH directed treatments. Whole-exome sequencing of predominantly liver fluke-negative, hepatitis virus-negative iCCAs by the Cancer Genome Atlas (TCGA) identified inactivating mutations in tumor suppressor genes, including *ARID1A*, *ARID1B*, *BAP1*, *TP53*, and *PTEN* as well as gain-of-function mutations in the oncogenes *IDH1*, *IDH2*, *BRAF*, and *KRAS* (143). Interestingly, focal losses of *CDKN2A*, encoding p16INK4A, which inhibits the cyclin-dependent kinases CDK4 and CDK6 were observed and at a substantially higher proportion (up to 15%) than reported previously (121, 141).

Receptor-tyrosine-kinase inhibitors (TKIs) Advanced stage solid malignancies, including iCCA are included into several selective and nonselective FGFR inhibitors for early phase clinical trials (143). Pan-FGFR inhibitors as NVP-BGJ398 and erdafitinib showed potential as well manageable safety profiles (144, 145). NVP-BGJ398 showed impressive results by a disease control rate of 82% (146) and tumor-activity results of erdafitinib from the ongoing phase II trial (NCT02699606) will be presented soon (147). Panatinib, another non-selective TKIs showed promising efficacy for FGFR2 fusions in patients with iCCA (148) and is currently evaluated in an ongoing phase II trial (NCT02265341). Several early phase I and phase II studies with selective FGFR-inhibitors including iCCA are ongoing like derazantinib (NCT01752920), TAS-120 (NCT02052778), Debio 1347 (NCT01948297), and INCB054828 (NCT02924376, NCT02393248).

Recently, an elegant experimental study shows that FGFR inhibition causes cell necrosis in human CCA cells, downregulating the expression of the myeloid cell leukemia 1 (Mcl1), a member of the Bcl-2 family of anti-apoptotic proteins. Necrosis is caused by the cellular depletion of Mcl1 within the mitochondrial matrix which impairs mitochondrial functions. Notably, cell death by necrosis induced by FGFR inhibition may be synergic for either chemotherapy, dampening intrinsic anti-apoptotic cellular resistance of CCA, or immunotherapy, by eliciting a strong immunological antitumor response (149).

Manipulation of CAFs

CAFs isolated from CCA patients show an enhanced susceptibility to apoptosis which results from an imbalance of Bcl-2 family members. Of note, the pro-apoptotic drug navitoclax, an inhibitor of Bcl-2, Bcl-xL, and Bcl-w, selectively induces apoptosis in CAFs and reduces tumor growth, as well as peritoneal and lymph node metastasis in a syngeneic rat model of CCA (15, 34). Moreover, A-1331852, a specific inhibitor for Bcl-xL, is able to induce apoptosis in activated fibroblasts and

reduces biliary fibrosis in a mouse model of primary sclerosing cholangitis (150), a pre-malignant condition of CCA. Thus, these data strongly support selective deletion of CAFs with Bcl-2 inhibitors as a therapeutic strategy in CCA.

Immunotherapy

Self-tolerance and protection of normal tissue during immune responses is maintained by immune checkpoints. These immune checkpoints are frequently altered by cancer cells to escape immune surveillance. Restoring the immune response to evoke anti-tumor immunity is a promising new approach in cancer therapy. As previously mentioned, two molecules are of special interest in this regard. CTLA-4 and PD-1/PD-L1 inhibitor are established for cancer immunotherapy. Studies of molecular phenotyping showed that immune checkpoint molecules are up-regulated in 45 % of BTCs (121). Further studies found overexpression of PD-1/PD-L1 in iCCA (122). Interestingly, tumors with immune checkpoint dysregulation showed less differentiated histology and more advanced tumor stage with worse outcome (127). However, data on immunotherapy in CCA are still scarce. The anti-PD-1 antibody pembrolizumab is under investigation in a phase II trial (NCT02628067). Preliminary data show promising efficacy in CCA with about 40% response rate. The PD-L1 inhibitor nivolumab has just been approved for HCC while data for CCA are still missing.

Besides immune checkpoint inhibitors, adoptive cell immunotherapy is a novel approach. Genetic reprogramming of autologous immune cells aims to enhance tumor cell recognition and anti-tumor immune response. Chimeric antigen receptor (CAR) T-cell therapy is one of the latest development approaches in this field. So far there are no adoptive immune cell therapies under clinical investigation for CCA.

Angiogenesis inhibitors

Although proangiogenic factors such as VEGF, are expressed in 50% of iCCA (151), the clinical relevance of angiogenesis inhibitors in iCCA remains controversial. A clinical phase II trial using bevacizumab, a humanized antibody targeting VEGF-A, in combination with gemcitabin and oxaliplatin (GEMOX), demonstrated a partial response in 41% of patients (152). Sorafenib, a tyrosine kinase inhibitor acting on VEGF receptors (VEGFR) and PDGFRs, reduced tumor growth in iCCA mouse models (153) but failed in clinical trials as single agent therapy or in combination with chemotherapy (154, 155). To further explain the disappointment with angiogenesis inhibitors it must be underlined that quite surprisingly, the main route of CCA dissemination through the lymphatic vascular system has not been considered yet for selective targeting (156). Interestingly, in a xenograft model of iCCA, targeting VEGFR-3 receptor (cognate of the main lymphangiogenic growth factor VEGF-C) markedly reduced tumor-associated lymphangiogenesis (34). Further investigations should also focus on the identification of CCA subgroups (e.g. patients with enhanced PDGF-BB level) who might benefit from angiogenesis inhibitors.

Conclusions

Studies in animal models and human samples have expanded the concept of the tumor microenvironment as a functional component central to tumorigenesis and tumor progression especially in epithelial cancers featuring an exuberant desmoplastic reaction. Within the multiple cell elements populating the microenvironment, new actors in particular from the immune system, have been added to the formerly characterized CAFs and TAMs, and make the interplay among them and with the tumor cells extremely intricate. Consequently, recent observations have argued against the original view that combinatorial interactions between different factors released in the tumor microenvironment boost the pervasive phenotype of cancer cells. Here we have dissected the pleomorphic functions of stromal and immune reactions in CCA (summarized in Figure 2). In this regard, LEL-CCA is paradigmatic of the protective role played by the immune milieu and this model will deserve strong attention by future studies aimed at testing efficacy of immunotherapy. On the other hand, very recent studies have further validated the pro-invasive functions exerted by stromal cells, showing that besides directly supporting the proliferative and invasive potential of cancer cells, CAFs provide them with a rich lymphatic vasculature instrumental for their early dissemination. Indeed, CAF depletion has led to significant anti-tumor effects in CCA. However, the considerable heterogeneity of CCA requires a multimodal, multiagent therapy that besides including tumor-promoting stromal cells, will gain traction from high throughput screening of target molecules and NGS-based stratification of patients, to identify and explore new effective and more personalized therapeutic approaches (157).

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Figure Captions.

Figure 1. Spatial relationships of different cell types in the tumor microenvironment of CCA. Immunohistochemistry for α-SMA (A), CD45 (B), CD34 (C), and dual immunohistochemistry for α-SMA (brown) and podoplanin (blue) (D) of formalin-fixed paraffin-embedded tissue sections obtained from surgical specimens of a patient with intrahepatic CCA undergoing liver resection. A) Cancer-associated fibroblasts identified by their immunoreactivity for α-SMA form a tight shell around the malignant bile ducts. B) Innate inflammatory cells expressing CD45 (neutrophils, macrophages, NK cells) are located in close vicinity to a large vascular space (*) consistent with their recruitment into the tumor microenvironment from the circulating compartment. C) Blood endothelial cells positive for CD34 are rarely observed nearby the neoplastic bile ducts, compared with D) the numerous podoplanin$^+$ lymphatic endothelial cells laying strictly adjacent to α-SMA$^+$ cancer-associated fibroblasts in between the tumoral ducts. A-C counterstained with DAPI. Original magnification: 200x.

Figure 2. Main features enabling the tumor microenvironment proficient to tumor growth and invasion in CCA. Compared with the normal stroma (upper side of the cartoon), the tumor microenvironment undergoes significant structural changes which profoundly affect the proliferative and invasive abilities of tumor cholangiocytes. The rupturing of the basement membrane (BM) allows invasive cholangiocytes to get access to a dense infiltrate comprising, among many cell types, activated cancer-associated fibroblasts (CAF), M2-polarized tumor-associated macrophages (TAM), regulatory T lymphocytes (Treg), and newly assembled lymphatic vessels through which tumor cells can early disseminate. A stiff extracellular matrix (ECM) provides support for the reciprocal interactions of the multiple cell types populating the tumor microenvironment.

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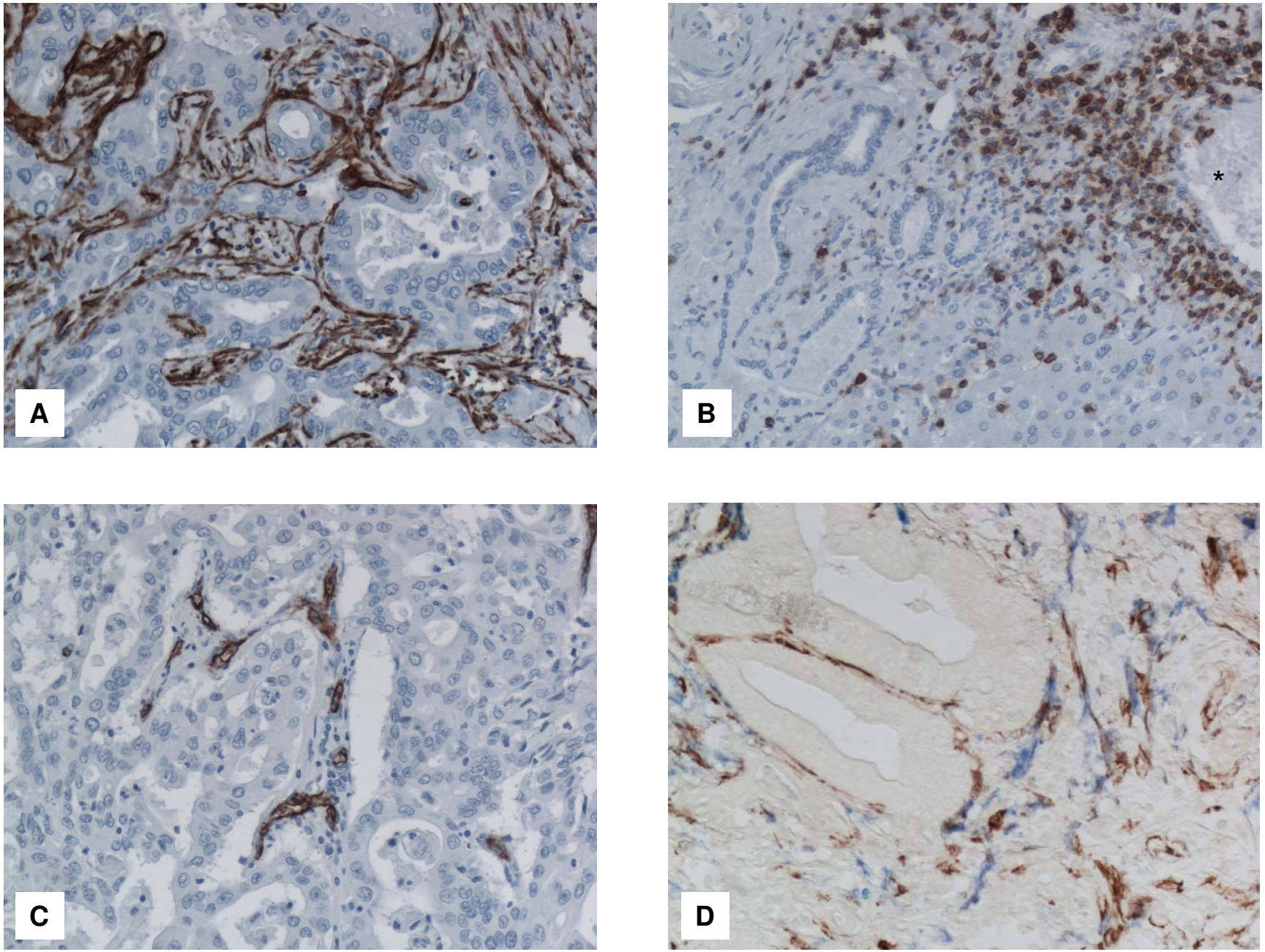

Figure 1

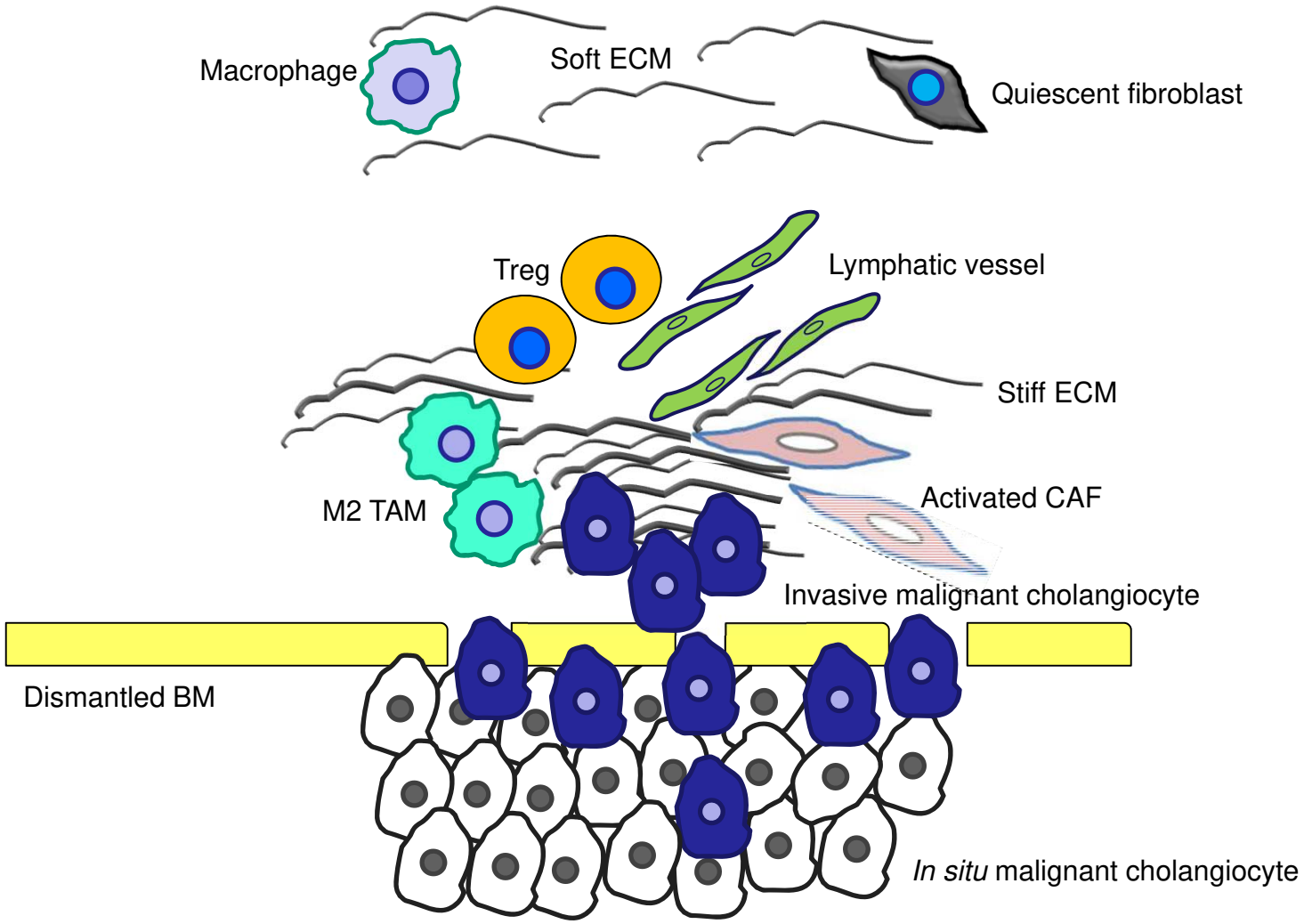
Macrophage
Soft ECM
Quiescent fibroblast
Treg
Lymphatic vessel
Stiff ECM
M2 TAM
Activated CAF
Invasive malignant cholangiocyte
Dismantled BM
In situ malignant cholangiocyte

Figure 2

Table 1. Tumor microenvironment-related biomarkers with prognostic relevance in CCA when increasingly expressed.

Biomarker	Site of expression	Biological significance	Prognostic correlation	Ref.
α-SMA	CAF	Cytoskeletal protein	Reduced survival	13,14
Podoplanin	CAF	Mucin-like transmembrane glycoprotein	Increased lymphatic metastasis	16
CD10	CAF	Cell surface metalloprotease	Increased distant metastasis	17
FAP	CAF	Membrane-bound serine protease implicated in ECM remodeling	Reduced survival and increased recurrence	38
MMP-1, MMP-2, MMP-3, MMP-9	CAF, TAM and tumor cells	Secreted matrix metalloproteases	Reduced survival	42,57
Periostin	CAF and ECM	TGF-β-inducible matricellular glycoprotein	Reduced survival and increased metastatic spread	45
Tenascin-C	ECM	Developmental matricellular glycoprotein	Increased tumor size, and lymphatic metastasis, reduced survival	46
Laminin	ECM	Developmental matricellular glycoprotein, major component of the basement membrane	Reduced survival	50
Osteopontin	ECM	Integrin-binding matricellular glycoprotein	Reduced survival	50
MAC387 (S100A8/9)	TAM	S100 calcium-binding proteins	Reduced survival	57
CD14/CD16	Circulating monocytes	CD14 – LPS co-receptor CD16 – FCγIII receptor	Reduced survival	58
CD15	TAN	Glycan determinant or Lewis x	Reduced survival	69
CD163	TAM	High affinity scavenger receptor for the hemoglobin-haptoglobin complex (M2 polarization)	Increased distant metastasis	77
TIE2	TAM	Receptor for angiopoietins	Improved survival	94
CD4/CD8	TIL (T helper and T cytotoxic lymphocytes)	CD4 – surface glycoprotein co-receptor of TCR and MHC-class II CD8 – surface glycoprotein co-receptor of TCR and MHC-class I	Improved survival, reduced lymphatic metastasis, reduced venous and perineural invasion	106-110

CD20	TIL (B lymphocytes)	Surface activated-glycosylated phosphoprotein expressed by B-cells through maturation	Low-grade differentiation tumors, improved survival	106
CD83	DC	Integral membrane protein belonging to the Ig superfamily involved in antigen presentation	Improved survival, reduced lymphatic metastasis	110
TGF-β	Tumor stroma	Pro-fibrogenic cytokine	Reduced survival, increased lymphatic and distant metastasis, increased recurrence	118
FoxP3	Treg and tumor cells	Member of the forkhead transcription factor family promoting immunosuppressive functions	Reduced survival, increased lymphatic metastasis, increased recurrence	111, 120
CTLA-4	Treg	Surface protein binding to CD80 on antigen-presenting cells to inhibit cytotoxic cells	Reduced survival, increased lymphatic metastasis	41
PD-1, PD-L1	Tumor cells	Immune check-point molecules	Reduced survival, increased lymphatic and distant metastasis	123, 126

CAF, cancer-associated fibroblasts

CTLA-4, cytotoxic T-lymphocyte antigen-4

DC, dendritic cells

ECM, extracellular matrix

PD-1, programmed cell death protein-1

PD-L1, programmed death-ligand 1

TAM, tumor-associated macrophages

TAN, tumor-associated neutrophils

TCR, T cell receptor

TIL, tumor-infiltrating lymphocytes

Treg, regulatory T lymphocytes

Table 2. Therapeutic strategies targeting tumor microenvironment in iCCA.

Cell compartment	Compound	Molecular target	Therapeutic Effects
CAF	Navitoclax	Bcl-2, Bcl-xL, Bcl-w	Reduction in both tumor growth, and peritoneal/lymph node metastasisation (animal models)
CAF	A-1331852	Bcl-xL	Reduction in biliary fibrosis (animal model)
TIL	Pembrolizumab	PD-1	Stimulation of immune system leading to reduction in tumor growth (human)
TIL	Nivolumab	PD-1	Stimulation of immune system leading to reduction in tumor growth (human)
Endothelial cell	Bevacizumab	Anti-VEGF	Reduction in tumor growth (human)
Endothelial cell	Sorafenib	VEGFR, c-KIT and PDGFR-α	Reduction in tumor growth (animal model), No effects (human)
Lymphatic endothelial cell	SAR131675	VEGFR-3	Reduction in tumor-associated lymphangiogenesis (animal model)

CAF, cancer-associated fibroblasts

c-KIT, proto-oncogene, receptor tyrosine kinase

PDGFR, platelet-derived growth factor receptor

PD-1, programmed cell death protein-1

TIL, tumor-infiltrating lymphocytes

VEGF, vascular endothelial growth factor

VEGFR, vascular endothelial growth factor receptor